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TRENDS IN RESEARCH ON *CRASSOSTREA VIRGINICA* AND ITS TWO PROTOZOAN PARASITES, *PERKINSUS MARINUS* AND *HAPLOSPORIDIUM NELSONI*

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Abstract

The protozoan parasites, *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX), are two important pathogens which have caused severe mortality in the eastern oyster, *Crassostrea virginica*, in the United States since 1950. This paper reviews and discusses the recent research on the diseases caused by these two parasites and focuses on: 1. the *in vivo* and *in vitro* interactions between *C. virginica* and *P. marinus*, 2. the physiological and biochemical characterization of the parasite, *P. marinus*, and 3. the development of DNA probes and polymerase chain reaction (PCR) primers specific for *H. nelsoni* or *P. marinus*.

Introduction

Disease has been a recognized problem in the cultivation of the eastern oyster, *Crassostrea virginica*. The first reported epizootic mortality occurred in Prince Edward Island, Canada. The etiological agent responsible for the mortality is now referred to as "Malpeque Bay disease". Since the outbreak of Malpeque Bay disease, a number of highly lethal infectious diseases of *C. virginica* and of other oyster species have been reported and described. Among these, the two protozoan parasites, *Perkinsus marinus* (cause Dermo disease) and *Haplosporidium nelsoni* (cause MSX disease), are two important pathogens which have caused severe mortality in eastern oysters in the United States since 1950s. *P. marinus* is predominant in water from the mid-Atlantic to the Gulf of Mexico. *H. nelsoni* is prevalent along the mid-Atlantic coast, and beginning in early 80s', its distribution has extended to Maine and Florida. Since the discovery of these two parasites in eastern oysters, numerous field and laboratory studies have been directed to the investigation of disease processes and transmission dynamics. Although the life cycle of *P. marinus* is not completely known, four life stages, merozoites/meronts, prezoosporangia, zoosporangia, and biflagellated zoospores have been identified and described (Perkins 1966, Perkins 1988, Perkins 1996). *P. marinus* is contagious and can be transmitted from infected to uninfected oysters (Ray and Mackin 1954, Andrews and Hewatt 1957, Mackin 1962). The three identified life stages, meront, prezoosporangia and biflagellated zoospore are infective stages (Chu 1996). The meront stage is more effective than the prezoosporangia stage in inducing infection (Volety and Chu 1994) and is believed to be the principal stage for transmitting disease in the field (Perkins 1988). The minimum dose required to infect oysters is 100 meronts or prezoosporangia per oyster via shell cavity inoculation. The life cycle of *H. nelsoni*, on the other hand, is not known. Only plasmodial stages of this parasite have been recognized in eastern oysters and no infective stages have been identified (Ford and Tripp 1996). Transmission of *H. nelsoni* among oysters has not been documented; an alternate or intermediate host has been hypothesized (Andrews 1968, Ford and Haskin 1982, Burreson 1988, Haskin and Andrews 1988). Beginning in late 80's, research has been directed to: (1) the *in vitro* and *in vivo* cellular interactions between *P. marinus* and the eastern oyster, (2) physiological and biochemical characterization of the parasite, *P. marinus*, and 3. developing DNA probes and/or polymerase chain reaction (PCR) primers specific for *H. nelsoni* and *P. marinus*. This presentation reviews and discusses some of the new findings in the above topics and future research options in the eastern oyster related to these two parasites.

In vitro and in vivo interactions between host-parasite

The outcome of the disease process is determined by interactions between the disease organism and the host and the environment. Temperature and salinity are two important factors limiting the distribution and progression of the diseases caused by these two agents in the field. *P. marinus* proliferates and develops rapidly between 20-30° C (*in vitro* and *in vivo*) and salinities greater than 10 ppt (Chu and Grenne 1989, Chu and La Peyre 1993, Ragone and Burreson 1993). *H. nelsoni* is active at temperatures above 10 °C (Ford and Trip 1996) and intolerant of salinities less than 10 ppt. *P. marinus* is persistent in salinity as low as 3 ppt and survives at temperatures as low as 4 °C.

To achieve a better understanding of the host defense mechanism, the defense-related hemocyte activities and their expression at a range of temperature and salinity conditions after challenge by *P. marinus* were investigated recently by Chu et al. (1993) and Chu and La Peyre (1993). Their studies revealed that: 1) oysters at higher temperatures had higher concentrations of circulating hemocytes, percentage of granulocytes and phagocytic capability, but did not have fewer or less intense *P. marinus* infections, and 2) oyster plasma lysozyme concentrations significantly correlated negatively with temperature and salinity.

The direct *in vitro* interactions between the host hemocytes and *P. marinus* have also been examined by the same laboratory. The *in vitro* interaction of *P. marinus* merozoites freshly isolated from infected oysters with hemocytes from eastern and Pacific (*Crassostrea gigas*) oysters were evaluated (La Peyre et al. 1995). Oyster hemocytes recognized and phagocytosed *P. marinus*. Electron microscopy examination revealed that merozoites were rapidly ingested by hemocytes from either oyster species but only some of the phagocytosed merozoites showed signs of degradation. No chemiluminescence (CL, a measurement of reactive oxygen intermediates) response was elicited from hemocytes of either oyster species. This suggests that intracellular killing of *P. marinus* is not mediated by reactive oxygen intermediates (ROIs), or the ROIs produced by oyster hemocytes are immediately scavenged by potential antioxidants of *P. marinus*. The

findings of suppression of CL generated by zymosan-stimulated hemocytes from eastern oysters by live laboratory cultured merozoites/meronts and their extracellular products (ECP) (Volety & Chu 1995) supports the latter hypothesis. The intracellular killings of *P. marinus* by eastern oyster hemocytes were further evaluated by measuring the killing index of live laboratory cultured *P. marinus* cells by hemocytes; it indicated that only a few individual oysters were capable of destroying the parasite intracellularly (Chu et al., unpublished data).

Information on the interaction between *H. nelsoni* and its host hemocytes is limited. Hemocytes from the eastern oyster did not phagocytose live *H. nelsoni* plasmodia; they phagocytosed only dead and moribund parasites (Ford et al. 1993).

In addition to warm temperatures and high salinity, environmental pollution could be a potential stressor responsible for elevated disease susceptibility and progression in oysters. The effect of contaminant exposure on the interactions between *P. marinus* and *C. virginica* was examined by Chu and Hale (1994). Exposure of oysters to the water soluble fraction (WSF) generated from sediments collected from the Elizabeth River, a heavily polluted subestuary of the Chesapeake Bay, USA, enhanced preexisting *P. marinus* infections and increased the oysters' susceptibility to experimentally induced infection, in a dose-dependent manner. The WSF contained predominantly low molecular weight polyaromatic hydrocarbons (PAHs) and heterocyclic compounds. Similarly, exposing oysters from a *P. marinus* enzootic area to Elizabeth River sediments, containing predominantly high molecular weight PAHs, elevated disease expression in oysters (Chu et al. unpublished data). Tributyltin (TBT, an additive to antifouling paint) exposure was also noted to intensify *P. marinus* infections and resulted in oyster mortality (Anderson et al. 1996, Fisher et al. 1995). Pollutant exposure may reduce disease resistance by causing physiological stress in the host or suppressing certain host defense mechanisms.

The above results imply that the oysters' cellular mechanisms may not be effective in defense against *P. marinus* and *H. nelsoni.* While the role of oyster lysozyme in oyster defense is unknown and remains to be examined, the higher plasma lysozyme in oysters at low temperature and salinity may offer an unfavorable environment for the development of the parasite and/or further weakening of parasite activity. *P. marinus* expression in oysters could be exacerbated by pollution, in addition to elevated temperature and salinity.

Physiological and Biochemical Characterization of P. marinus

Because of the ineffectiveness of *C. virginica* 's defense against *P. marinus* (Chu & La Peyre 1993, Chu et al. 1993), scientists, in the search to control *P. marinus,* studied factors responsible for the virulence of this parasite starting 1993.The recent establishment of *in vitro* cultures of *P. marinus* (Gauthier & Vasta 1993, Kleinschuster & Swink 1993, La Peyre et al. 1993) promotes such efforts and open an avenue to characterize this parasite physiologically and biochemically, although laboratory cultured *P. marinus* cells appeared to be less infective/pathogenic than those freshly isolated from infected oyster tissues (Chintala et al., 1995; Perkins, unpublished data; Volety and Chu, unpublished data). Analysis of *P. marinus* cells and extracellular products revealed the presence of various enzymes, such as acid phosphatase (AP, Volety and Chu, In press), triacylglycerol hydrolase, phospholipase A^2 (Chu et al. unpublished data), multiple proteases, glycosidases (La Peyre et al. 1995, La Peyre 1996), superoxide dismutase (SOD, Ahmed et al. 1997, Wright & Vasta 1997, Chu and Armknechet, unpublished data). These enzymes may contribute to virulence of *P. marinus.* Proteases from *P. marinus* digested a variety of proteins, including oyster plasma protein (La Peyre et at. 1995). AP and SOD are hypothesized to be the antioxidants for CL suppression (Volety and Chu 1995, Ahmed et al. 1997, Wight & Vasta 1997). Both extracellular AP and protease increase with the increasing growth rate of the parasite (Volety & Chu , In press; La Peyre 1996).

Host lipids play a unique role for long-term survival and life cycle completion in endogenous parasites (Furlong 1991). The work on the lipid biochemistry of *P. marinus* showed that *P. marinus* actively takes up lipids from its culture medium and is capable of synthesizing new phospholipids using exogenous lipid sources (Chu et al., unpublished data). Lipolytic enzymes probably play a significant role in acquiring lipids from the oysters. *P. marinus* has much higher triacylglyerol hydrolase and phospholipase A^2 than oyster hemocytes and plasma (Chu et al., unpublished data).

The above results suggest that this parasite possesses virulence factors such as APs, SOD, proteases, and lipolytic enzymes, through which the parasite effectively evades the host's defense mechanism.

DNA Probes and Polymerase Chain Reaction (PCR) Primers specific for P. marinus and H. nelsoni

Histological examination and tissue assays are the traditional diagnostic assays for *H. nelsoni* and *P. marinus* respectively (Ford and Trip 1996). The disadvantages of these assays are that they are not sensitive enough to detect early and latent infections and the assay procedures are quite time consuming. Nonlethal hemolymph assays have been developed for both parasites (Gauthier and Fisher 1990, Morrison et al. 1992), but they are not as accurate as histological and tissue assays. The immunological probes (Burreson 1988, Dungan and Roberson 1993) using polyclonal antibodies against *H. nelsoni* or *P. marinus* are more sensitive. However, these polyclonal antibodies do not differentiate *P. marinus* or *H. nelsoni* from other *Perkinsus* or *Haplosporidium* species. Thus, more sensitive and specific diagnostic assays are required for detection of these two parasites. A DNA probe specific for *H. nelsoni* (oligonucleotide designated MSX 1347) has been developed recently (Stokes and Burreson 1995). Two oligonucleotides specific to *H. nelsoni* (MSX-A and MSX-B) have also been identified, used for PCR amplification, and tested for specificity and sensitivity in detecting *H. nelsoni* (Stokes et al. 1995). The DNA probes and PCR primers are more sensitive and specific than the traditional histological, hemolymph, and immunological assays. Currently, scientists are working on developing DNA probes and PCR primers specific for *P. marinus* (Marsh &

Vasta 1995, Wright et al. 1997, Yarnall et al.1997). The establishment of these molecular tools will allow scientists not only to investigate the life cycle of *H. nelsoni* and fill the missing links in *P. marinus* life cycle, but to examine the phylogenies of these two parasites. The investigation of the phylogeny of *H. nelsoni* (Siddall et al. 1995) and *P. marinus* (Goggin and Barker 1993), as well as the population genetic structure of the latter is currently in progress.

Summary

Researches on these two parasites have evolved to a stage of physiological, biochemical, and molecular approaches. Studies on these aspects will be continued. By understanding the basic mechanisms of the host-parasite interactions and their expressions in different environments, we may find ways to promote the host defense and to control the parasites' virulence. *H. nelsoni*'s life cycle is completely unknown. Neither the infective stage has been identified nor has *in vitro* culture of this parasite been established. This has hindered the laboratory investigations of its interaction with the eastern oyster and its disease processes and transmission dynamic in oysters. The establishment of molecular probes for *H. nelsoni* hopefully will resolve the life cycle of this parasite. When molecular probes become routine diagnostic assays for these two parasites, it will allow better management of our oyster resources.

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